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Scalable Production of iPSC-Derived Human Neurons to Identify Tau-Lowering Compounds by High-Content Screening

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SUMMARY

Lowering total tau levels is an attractive therapeutic strategy for Alzheimer's disease and other tauopathies. High-throughput screening in neurons derived from human induced pluripotent stem cells (iPSCs) is a powerful tool to identify tau-targeted therapeutics. However, such screens have been hampered by heterogeneous neuronal production, high cost and low yield, and multi-step differentiation procedures. We engineered an isogenic iPSC line that harbors an inducible neurogenin 2 transgene, a transcription factor that rapidly converts iPSCs to neurons, integrated at the AAVS1 locus. Using a simplified two-step protocol, we differentiated these iPSCs into cortical glutamatergic neurons with minimal well-to-well variability. We developed a robust high-content screening assay to identify tau-lowering compounds in LOPAC and identified adrenergic receptors agonists as a class of compounds that reduce endogenous human tau. These techniques enable the use of human neurons for high-throughput screening of drugs to treat neurodegenerative disease.

INTRODUCTION

The microtubule-associated neuronal protein tau stabilizes microtubules and mediates axon outgrowth and axonal transport. Abnormal tau is strongly implicated in Alzheimer's disease (AD) and other neurodegenerative tauopathies (Wang and Mandelkow, 2016). Although intraneuronal aggregates of insoluble tau fibrils, known as neurofibrillary tangles, are a hallmark of tauopathies and correlate with cognitive decline in AD (Nelson et al., 2012), soluble tau may also play a key pathogenic role (Brunden et al., 2008; Spires-Jones et al., 2011). In *Drosophila* and mouse models, overexpression of wild-type human tau induces neurodegeneration (Wittmann et al., 2001), axonopathy (Spittaels et al., 1999), and extensive cell death (Andorfer et al., 2005) independently of tangle formation. In two regulatable tauopathy mouse models, suppressing soluble tau expression resulted in memory recovery (Santacruz et al., 2005; Sydow et al., 2011) and stabilized neuron numbers (Santacruz et al., 2005) without reducing the level of neurofibrillary tangles, suggesting that soluble forms of tau promote neurodegeneration. Lowering endogenous tau levels reduces amyloid β (A β)-induced behavioral deficits in AD mouse models (Roberson et al., 2007; Vossel et al., 2010), and lowering total tau levels by inhibiting tau acetylation or

phosphorylation rescues tau-related memory deficits in PS19 transgenic mice (Lasagna-Reeves et al., 2016; Min et al., 2015). Since tau knockout mice appear to be cognitively normal, lowering total tau levels in neurons appears to be safe and will likely have a high therapeutic index (Morris et al., 2013). Thus, soluble tau is a promising therapeutic target. However, identifying selective, non-toxic tau-lowering compounds has proven to be difficult (Gruninger, 2015).

Cell-based "phenotypic" high-throughput screening (HTS) is a powerful unbiased tool to identify gene targets or small-molecule compounds exerting desired effects. However, HTS requires large numbers of cells and has been largely restricted to immortalized human neuronal lines, such as neuroblastoma SH-SY5Y (Jain et al., 2012) and glioma H4 (Albrecht et al., 2004) cells, or non-neuronal lines, such as HeLa cells (Fatokun et al., 2013). Since these cells differ physiologically from post-mitotic neurons, hits identified in these cells might not work in neurons. This may be particularly true for tau, a neuronal protein that is abundant in axons but is mainly expressed in the cytosol in non-neuronal cells (Uberti et al., 1997). Rodent primary neurons are more physiologically relevant, but challenges in scalability preclude their use for HTS, and certain compounds may differ in activity between human and rodent cells.

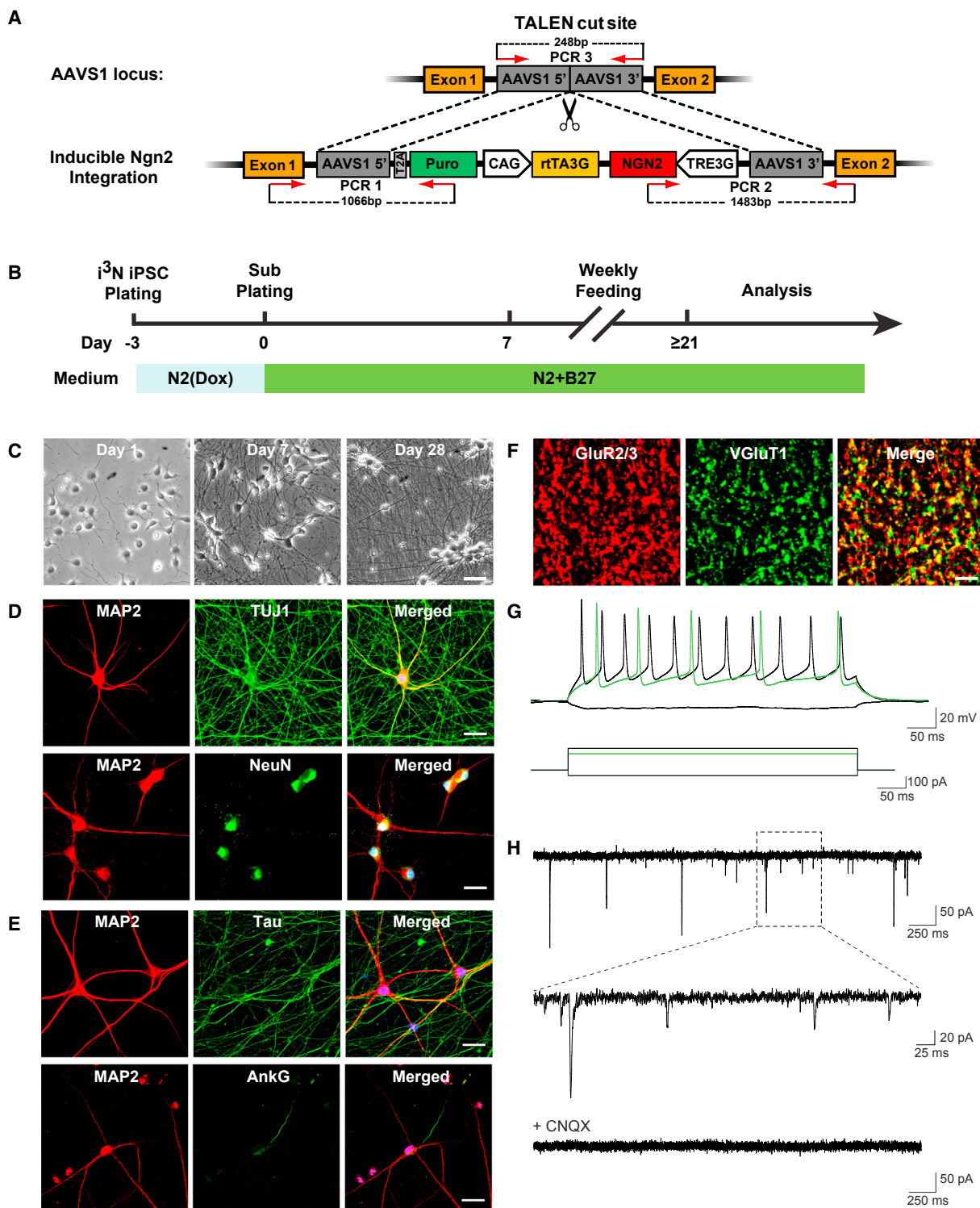


Figure 1. Engineering of i³N iPSCs and Generation of Homogeneous Functional Glutamatergic Neurons by a Simplified Two-Step Procedure

(A) Schematic of the targeting of the AAVS1 locus with pUCM.Puro-CAG.rtTA3G-TRE3G.Ngn2 donor vector by TALEN-mediated integration. The third-generation doxycycline-inducible reverse transcriptional activator (rtTA3G) is driven by the CAG promoter and followed by rbGlob polyA tail. Mouse *Ngn2* is driven by the tet response element (TRE3G) and followed by SV40 polyA tail. It is oriented tail-to-tail with

(legend continued on next page)



Human induced pluripotent stem cells (iPSCs) are a promising alternative because they can be used to generate large numbers of subtype-specific human neurons that are relevant to neurodegenerative disease. However, iPSC-derived neurons currently have limited utility in HTS assays (D'Aiuto et al., 2014), as traditional differentiation methods are difficult to scale up and usually yield a heterogeneous population of neurons and glia-like cells over a protracted timeline (Muratore et al., 2014; Nicholas et al., 2013). More homogeneous neuronal populations can be produced by overexpressing pro-neuronal transcription factors (Chanda et al., 2014; Pang et al., 2011). Neurogenin 2 (NGN2)-induced neurons from various human embryonic stem cell and iPSC lines show robust morphological, transcriptional, and functional homogeneity (Busskamp et al., 2014; Zhang et al., 2013). However, this method has shortcomings for HTS. First, it entails a labor-intensive multi-step differentiation procedure that is difficult to apply to microplates. Second, it is subject to cell-to-cell and well-to-well variability due to different viral infection and puromycin selection rates, uneven cell distribution, which might affect cell survival and image quantification, and experiment-to-experiment variability due to differences in viral titers and qualities of primary mouse glia from different batches. Third, it is costly to scale up.

In this study, we engineered a clonal iPSC line that stably harbors a doxycycline-inducible mouse *Ngn2* transgene at an adeno-associated virus integration site 1 (AAVS1) safe-harbor locus. This integrated, inducible, and isogenic *Ngn2* iPSC line (i^3 N) can be differentiated into functional glutamatergic cortical neurons by a simplified two-step differentiation protocol. We developed a robust high-content screening (HCS) assay to identify tau-lowering compounds and discovered compounds that target adrenergic receptor (AR) pathways to lower endogenous human tau.

RESULTS

Engineered iPSCs for Scalable Production of Homogeneous Excitatory Neurons

Lentivirus-mediated NGN2 expression induces rapid differentiation of iPSCs into excitatory neurons (Zhang et al., 2013). To avoid viral transduction-induced toxicity and variability in NGN2 expression, we engineered isogenic iPSC lines with an integrated *Ngn2* expression cassette. A doxycycline-inducible *Ngn2* transgene was integrated into the AAVS1 safe harbor of a well-characterized control human iPSC line (WTC11) (Miyaoaka et al., 2014) by TALEN-mediated integration of a donor cassette containing a puromycin-resistance gene (Figure 1A). Six puromycin-resistant clones were picked, and integration of the *Ngn2* transgene into the AAVS1 locus was confirmed with two sets of primers (PCR1 and PCR2) (Figures 1A and S1A). Transgene integration into both alleles was confirmed by the absence of the wild-type allele, determined by a third set of primers (PCR3) (clones 1 and 4). These iPSC clones have isogenic, integrated, and inducible NGN2 expression (i^3 N); neurons derived from them are called i^3 Neurons. Further characterization of clone 1 in the absence of doxycycline showed homogeneous expression of the pluripotency markers OCT4, SOX2, and TRA-1-81, indicating no leakage of NGN2 expression (Figure S1B). The cells also had a normal karyotype (Figure S1C).

i^3 N iPSCs could be differentiated into functional excitatory neurons by the published multi-step differentiation protocol. To overcome the poor scalability and reproducibility of this procedure when adapted to the HTS platform, we established a simplified two-step protocol (Figure 1B). After doxycycline induction and subplating, pre-differentiated i^3 Neurons became post-mitotic, and

rtTA3G. Orange boxes are exons of the PPP1R12C gene; gray boxes are regions of homology. PCR1 and PCR2 primers are used for 5' and 3' junction PCR screening and generate 1.1-kb and 1.5-kb PCR products, respectively. PCR3 primers (product size, 248 base pairs) are used to detect the nonintegrated allele at the AAVS1 locus.

(B) Flow diagram of the two-step procedure for generating i^3 Neurons.

(C) Representative phase-contrast images during the differentiation of i^3 Neurons. The timeline is the same as shown in (B). Scale bar, 50 μ m.

(D) Representative images showing immunocytochemical staining for the pan-neuronal marker MAP2, β III tubulin (TUJ1 antibody), and NeuN in i^3 Neurons after 4 weeks of differentiation. Nuclei were labeled by Hoechst. Scale bar, 25 μ m.

(E) Representative images of immunocytochemical staining of mature 8-week-old i^3 Neurons show tau enrichment (detected with HT7) in an axon identified by the axon initiation segment marker ankyrin G (AnkG). Nuclei were labeled by Hoechst. Scale bar, 25 μ m.

(F) Representative confocal images of i^3 Neurons showing immunolabeling of postsynaptic GluR2/3 containing AMPA-type glutamate receptors (red) and the presynaptic vesicular glutamate transporter VGLut1 (green). The colocalization of GluR2/3 and VGLut1 puncta marks glutamatergic synapses formed between i^3 Neurons. Scale bar, 5 μ m.

(G) Representative traces of action potentials evoked by 500-ms current step injections at just above the firing threshold (green trace) and at a higher firing frequency (black trace).

(H) Spontaneous excitatory postsynaptic currents recorded from an i^3 N neuron (top) were blocked by CNQX, an AMPA receptor antagonist (bottom).

See also Figures S1 and S2.



exhibited neuron-like morphology in less than 7 days and mature neuronal morphology within 3–4 weeks in the absence of glia (Figure 1C). Differentiated neuron markers β III tubulin (TUBJ1), microtubule-associated protein 2 (MAP2), and neuronal nuclei antigen (NeuN) were detected in i^3 Neurons after 4 weeks of maturation (Figure 1D). The absence of Olig2-positive oligodendrocytes or glial fibrillary acidic protein (GFAP)-positive astrocytes confirmed the purity of the neuronal population (Figure S2A).

In 8-week-old i^3 Neurons, tau was abundant in axons identified by the presence of a single axonal initial segment (AIS), as detected with ankyrin G staining (Kordeli et al., 1995) (Figure 1E). More than 90% of i^3 Neurons contained AIS, supporting the notion that they exhibit mature polarity by 8 weeks (Figure S2B). Tau expression had little overlap with MAP2, a dendritic marker (Figure 1E). Both three-repeat (3R) and four-repeat (4R) tau were detected (Figure S2C). Tau in i^3 Neurons were also highly phosphorylated, compared with healthy human brain (Figure S2D). Importantly, all i^3 Neurons expressed vesicular glutamate transporter 1 (VGLUT1), a marker of glutamatergic neurons, and were GABA negative, indicating a homogeneous population of excitatory glutamatergic neurons (Figure S2E). The punctate distribution of synapsin-1 staining along the processes revealed abundant synapse formation (Figure S2F). When co-cultured with glia, i^3 Neurons had mature synapses that contained juxtaposed pre- and post-synaptic markers of glutamatergic synapses (Figure 1F). Whole-cell patch-clamp recordings showed action potential firing in response to current injections (Figure 1G). Spontaneous postsynaptic currents detected in i^3 Neurons were blocked by CNQX, an AMPA receptor antagonist, confirming functional glutamatergic synaptic transmission (Figure 1H).

i^3 Neurons Cultured in Microplates Show Homogeneous Gene Expression with Low Variability

Two key determinants of reliable HTS are scalability and minimal well-to-well variability. We were able to produce large quantities of i^3 Neurons via i^3 N iPSC proliferation and a pre-differentiation step. Subsequent subplating enables i^3 Neurons culture in 96- and 384-well microplates with even cell distribution (Figure 1B). To determine whether i^3 Neurons homogeneously differentiate in microplates, we quantified the expression of multiple neuronal and glial genes of 4-week-old cells from 12 randomly selected wells of a 96-well plate by RT-qPCR (Figure 2). Consistently high levels of pan-neuronal genes, AMPA receptor genes, glutamate transporter genes, synaptic genes, and *CUX1*, a marker of cortical layer 2/3 neurons, were observed in all 12 wells, suggesting a uniform differentiation of glutamatergic cortical neurons. In addition, all the i^3 Neurons showed low expression of two markers of neural

progenitor cells, *PAX6* and *NESTIN*, indicating that they were fully differentiated. *Ngn2* was undetectable, as expected in the absence of doxycycline. No markers of glial cells or of GABAergic neurons (e.g., *GAD65/67*) were detected, but the GABA receptors *GABRA2* and *GABRB1* were expressed at low levels. Thus, i^3 Neurons can be cultured in microplates with low well-to-well variability and are suitable for HTS.

Optimization of an HCS Assay to Screen for Tau-Lowering Compounds

Lowering tau levels has emerged as a key therapeutic opportunity in AD. To our knowledge, no HCS targeting endogenous tau has been performed in post-mitotic human neurons. Because i^3 Neurons are scalable and highly homogeneous, they are an ideal system to screen for tau-targeted therapeutics. Taking advantage of the highly specific anti-human tau antibody HT7, we set out to develop a 384-well format HCS assay to detect endogenous tau levels in i^3 Neurons (Figure 3A). Total tau levels were determined by immunoreactivity of HT7 normalized to β III tubulin intensity in a corresponding well (HT7/TUBJ1) (Figure 3B). The background signal, measured in wells omitting HT7, was more than 10-fold lower than those with HT7 (Figures 3B and S3A). The optimal seeding density was 2,000 cells/well (Figure S3A). Neuronal health was judged from neurite total length (Figure S3B) and valid nucleus count (Figure S3C), two highly correlated health parameters (Figure S3D) that are widely used (Harrill et al., 2013; K Hancock et al., 2015). Treatment with a tau-specific siRNA reduced the total tau levels in a dose-dependent manner (Figures 3C and 3D), whereas increased total tau levels were detected when i^3 Neurons were infected by AAV human tau (Figures S3E and S3F), confirming assay specificity and sensitivity. Salicylate, YM-01, and methylene blue reduce total tau levels (Abisambra et al., 2013; Hosokawa et al., 2012; Min et al., 2015). All three compounds reduced the HT7/TUBJ1 signal in a time-dependent manner accompanied by a mild yet significant reduction of neurite total length (Figure 3E). The Z' factor, a measure of the assay response window (Zhang et al., 1999) and determined by values of control and hTau siRNA, was 0.41, supporting the robustness of the HCS assay.

Identification of Tau-Lowering Compounds via HCS

Next, we used the HCS assay to screen LOPAC (Library of Pharmacologically Active Compounds) for tau-lowering compounds. This library contains 1,280 bioactive small molecules, including inhibitors, receptor ligands, marketed drugs, and pharmaceutically relevant structures, that affect most signaling pathways and cover major drug target classes. Since overwhelming majority compounds did not change tau levels (Figure 4A), we used sample compounds

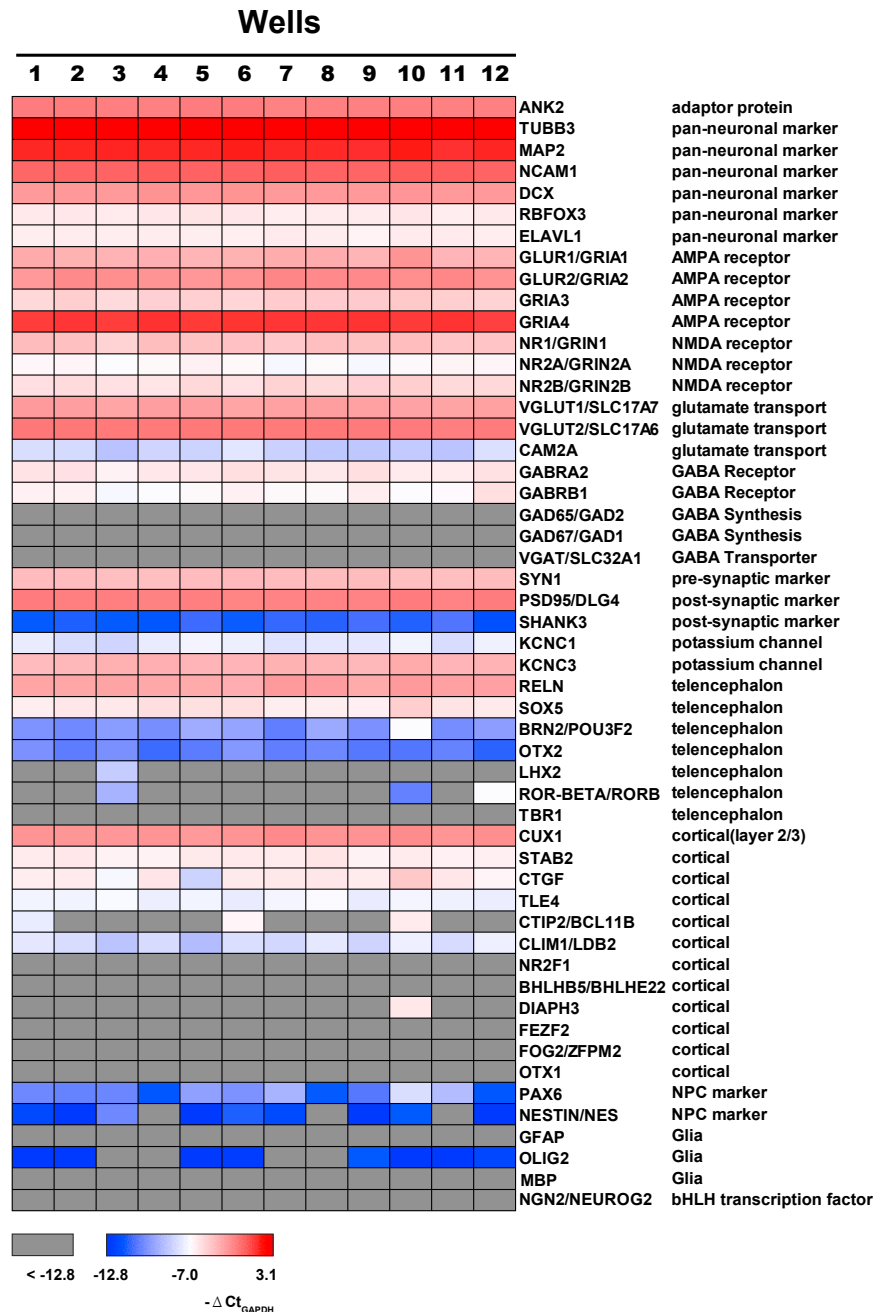


Figure 2. *i*³Neurons Show Homogeneous Expression of Glutamatergic Cortical Neuronal Genes

Heatmap of RT-qPCR analysis of expression levels of genes listed on the right. Expression levels are normalized to housekeeping gene *GAPDH* (expressed as $-\Delta Ct$) and color coded as shown. mRNA was harvested from 12 random wells of 4-week-old *i*³Neurons cultured in a 96-well plate. See also Table S1.

as their own control and calculated Z scores based on LOPAC compounds on each plate (Brideau et al., 2003). Compounds that changed tau (as judged from the HT7/TUJ1 ratio) were defined as hits if their Z scores were greater than 3 or less than -3, a stringent cut-off correlates to a p value of 0.00135 (Zhang et al., 2006). All hits were then ranked by neuronal health parameters, including neurite total length (Figure 4B) and valid nucleus count (Figure 4C).

The top two tau-lowering hits that show least cytotoxicity, measured by neurite total length and valid nucleus

count, are two functionally related AR agonists, moxonidine and metaproterenol (Figures 4B and 4C). To validate these hits, we adapted a sensitive human tau ELISA that uses HT7 as the capture antibody and Tau5 as the detection antibody (Meredith et al., 2013). The dynamic range of the assay was >3,000, and the detection limit was ~10.7 pg/mL (Figure 5A). This assay readily detected siRNA-induced reduction of endogenous human tau by >50% (Figure 5B). We then confirmed that moxonidine, clonidine (a moxonidine-related adrenergic agonist), and

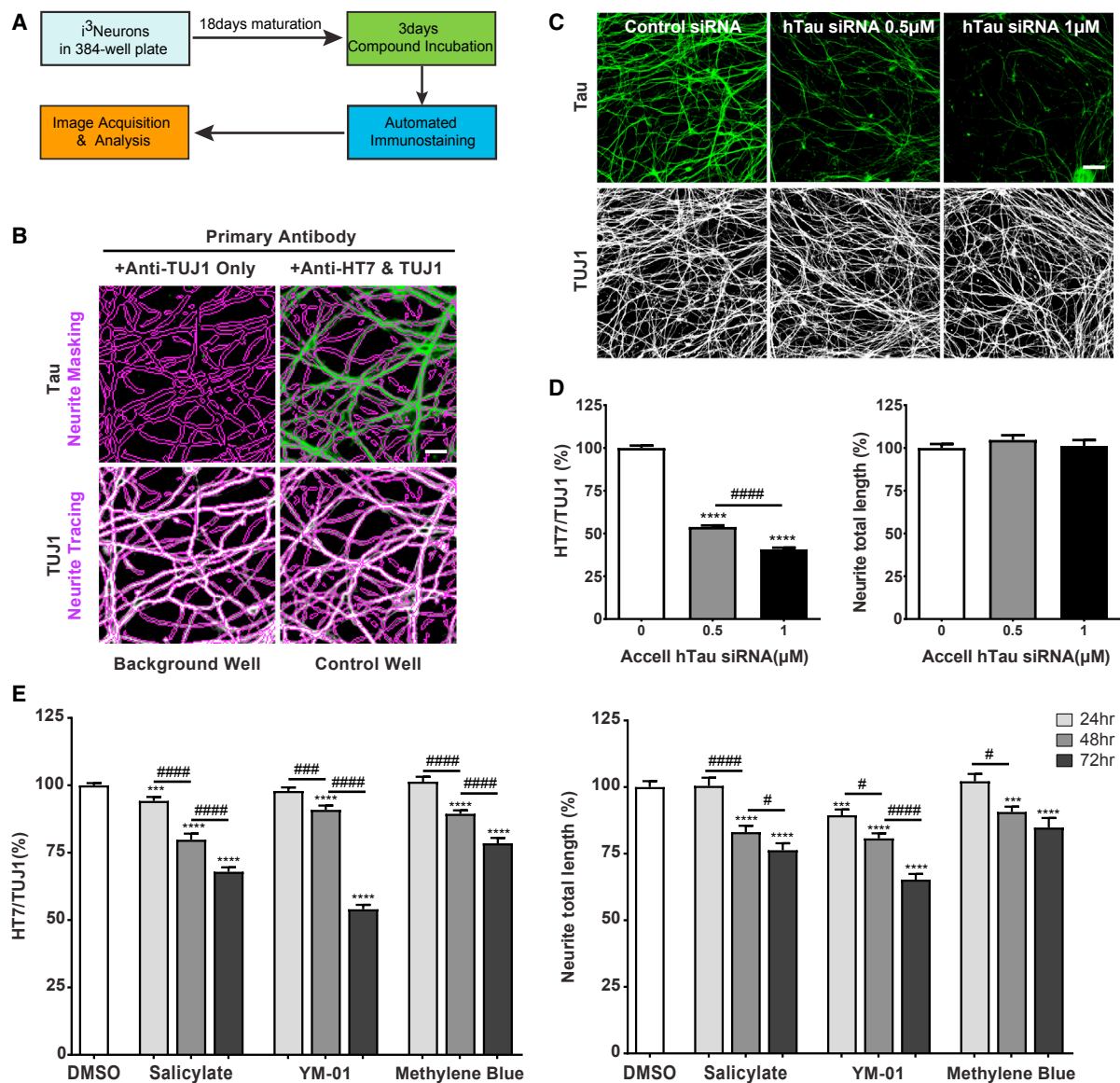


Figure 3. Development and Validation of an HCS Assay to Detect Tau Levels in *i*³Neurons

(A) Schematic of the HCS assay optimized to measure cellular tau levels in neurons treated with small-molecule compounds.

(B) Representative fluorescence high-content images showing tau (green) and β III tubulin (white) channels from a background well (left, anti-TUJ1 only) and a control well (right, anti-HT7 and TUJ1). Neurite regions (purple) were traced according to the TUJ1 channel and were applied to the tau channel with the neuronal profiling module of Cellomics software. Scale bar, 10 μ m.

(C) Representative fluorescence high-content images showing tau (green) and β III tubulin (white) channels from *i*³Neurons after 7 days of treatment with control siRNA or human tau siRNA (0.5 or 1 μ M). Scale bar, 50 μ m.

(D) Automated quantification of human tau levels (left) and neurite total length (right) from *i*³Neurons treated with human tau siRNA. Data are from three independent experiments, total N = 42 per treatment; values are means \pm SEM relative to control siRNA. ****p < 0.0001 compared with control siRNA, STATA mixed model. #####p < 0.0001, STATA mixed model.

(E) Automated quantification of human tau levels (left) and neurite total length (right) from *i*³Neurons treated with 5 mM salicylate, 1 μ M YM-01, or 1.5 μ M methylene blue for 24–72 hr. Data are from three independent experiments, total N = 42 per treatment; values are means \pm SEM relative to DMSO. ***p < 0.001, ****p < 0.0001, compared with DMSO, STATA mixed model; #p < 0.05, #####p < 0.0001, comparison between three time points within each compound treatment, STATA mixed model.

See also [Figure S3](#).

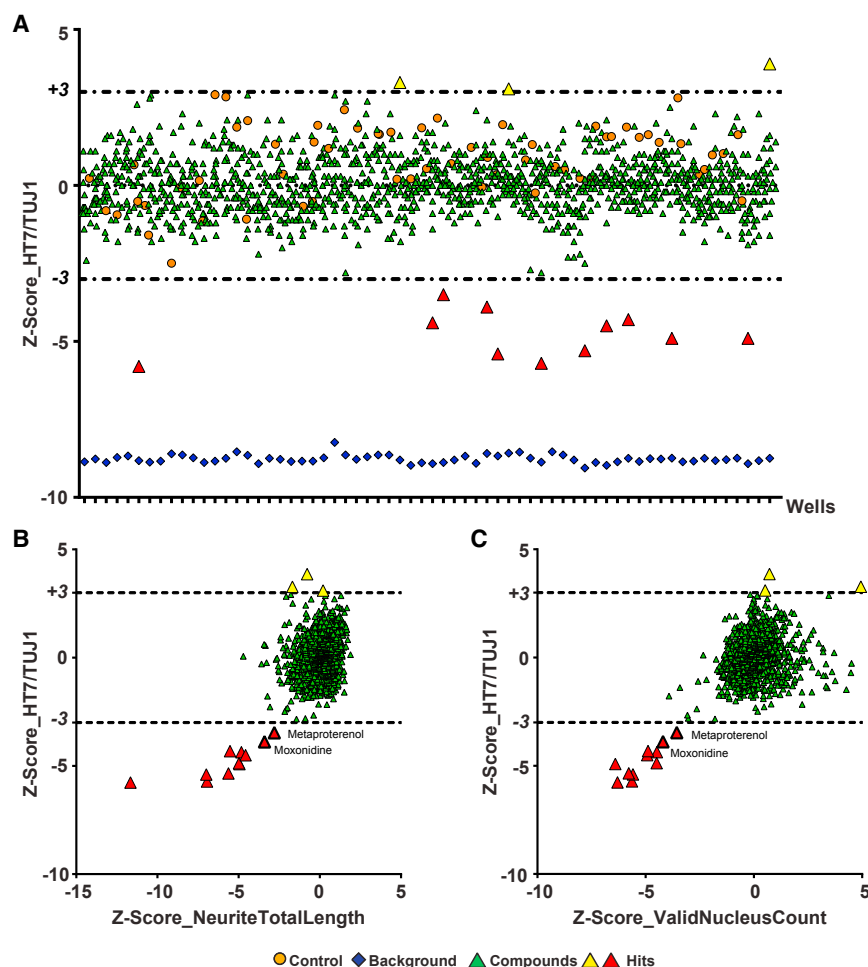


Figure 4. Primary Screening and Hit Selection from LOPAC

(A) Overview of automated quantification of human tau levels from i³Neurons incubated with compounds from LOPAC at 10 μ M for 3 days (green triangles). The library was distributed to three and one-half 384-well plates; each plate contains 16 control wells (DMSO, orange circles) and 16 background wells (blue diamonds). Results are shown as the Z score, calculated based on LOPAC compounds on each plate. Compounds above (yellow triangles) and below (red triangles) the cut-offs of +3 or -3 (dotted lines) are considered hits.

(B and C) Hits are ranked by neurite total length (B) and valid nucleus count (C). Results are shown as Z score, calculated based on LOPAC compounds on each plate. Moxonidine and metaproterenol are the top two tau-lowering hits from both rankings.

metaproterenol reduced tau levels in a dose-dependent manner. None of the three compounds induced a significant loss of β III tubulin, as measured with a β III tubulin ELISA, indicating a lack of toxicity (Figures 5C–5E).

Additional pharmacologic modulators of α - and β -AR activity were used to further confirm the involvement of α - and β -adrenergic signaling in modulating endogenous human tau levels. i³Neurons were treated with nonselective α - or β -AR agonists (dexmedetomidine or isoproterenol) and their respective antagonists. Activation of α - or β -ARs significantly reduced endogenous human tau levels, and inhibition by nonselective antagonists caused tau accumulation (Figures 5F and 5G). Moreover, the tau-lowering effects of moxonidine and metaproterenol were abolished by pre-incubation with the corresponding antagonists (atipamezole and propranolol, respectively) (Figures 5F and 5G). The ability of α - or β -AR agonists to reduce endogenous tau levels was further confirmed in an independent line of i³Neurons (Figures S4A and S4B). Our results showed that activation of α - and β -adrenergic

signaling pathways could represent tau-lowering therapeutic strategies in human neurons.

DISCUSSION

In this study, we integrated a doxycycline-inducible mouse *Ngn2* cassette into the AAVS1 locus and established iPSC lines that can be converted efficiently to a uniform population of glutamatergic neurons by a simplified two-step protocol. We then developed a robust, scalable, and simple HCS assay and used it to identify compounds that lower tau in post-mitotic human neurons. This proof-of-principle screen identified AR agonists as a class of compounds that decrease endogenous human tau levels.

i³Neurons have several features that make them suitable for HTS, particularly imaging-based HCS. First, the population of differentiated neurons is homogeneous, which may reflect the uniform genetic background of the clonal i³N iPSCs. As all of the iPSC-derived cells are neurons,

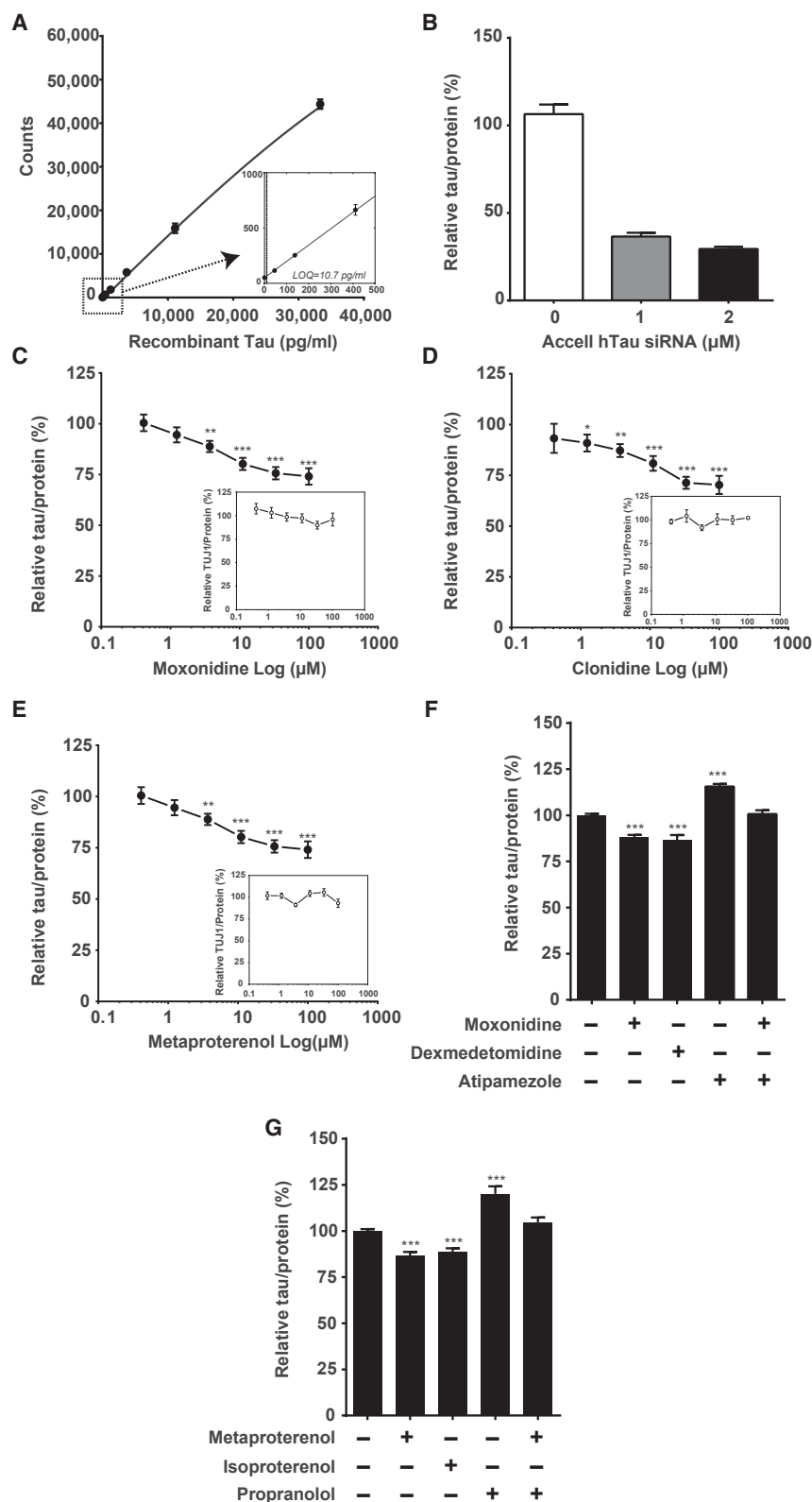


Figure 5. Activation of α - and β -AR Reduces Total Tau Levels in i^3 Neurons

(A) Representative calibration curve of HT7-Tau5 ultra-sensitive human tau ELISA. Inset shows the assay's limit of quantification (LOQ).

(B) 7-day incubation of human tau siRNA significantly lowered total tau levels in i^3 Neurons. Human tau levels were quantified by HT7-Tau5 ELISA and normalized to protein level. Values are means \pm SEM relative to control siRNA. Data are from one experiment, $N = 6$ wells per treatment.

(C–E) Concentration-response curve of moxonidine (C), clonidine (D), and metaproterenol (E) determined by HT7-Tau5 ELISA. Insets show the β III tubulin level for each concentration as determined by β III tubulin ELISA. Both tau and β III tubulin levels are normalized to protein levels. Values are means \pm SEM relative to DMSO control. Data are from four independent experiments performed in triplicate (C, $N = 12$ per concentration) and three independent experiments performed in triplicate (D and E, $N = 9$ per concentration). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; STATA mixed model.

(F and G) 3-day incubation with moxonidine (30 μ M) and the α -AR agonist dexmedetomidine (100 μ M) (F) or metaproterenol (30 μ M) and the β -AR agonist isoproterenol (30 μ M) (G) significantly reduced total tau levels in i^3 Neurons. The α -AR antagonist atipamezole (100 μ M) (F) and the β -AR antagonist propranolol (50 μ M) (G) increased total tau levels and abolished the tau-lowering effect of moxonidine (F) or metaproterenol (G), respectively. Human tau levels were quantified by HT7-Tau5 ELISA and normalized to protein level. Values are means \pm SEM relative to DMSO control. Data are from three independent experiments performed in triplicate (F, $N = 9$ per treatment) and four independent experiments performed in triplicate (G, $N = 12$ per treatment) *** $p < 0.001$, STATA mixed model.

See also [Figure S4](#).



sensitivity and specificity of HTS screens is increased. Other safe-harbor loci besides the AAVS1 locus (e.g., citrate lyase beta-like) can be used in human iPSCs (Cerbini et al., 2015). By targeting multiple safe-harbor sites with P2A or IRES elements, it may be possible to integrate multiple inducible transcription factors to differentiate iPSCs into other neuronal subtypes, such as dopaminergic neurons (Amamoto and Arlotta, 2014) or motor neurons (Hester et al., 2011), for use in HTS. Second, our simplified glia-free differentiation protocol overcomes hurdles, such as scalability, variability, complexity, and cost, that previously hindered the use of iPSC-derived neurons in drug discovery pipelines. For example, instead of generating and freezing large quantities of neuronal precursor cells, our approach involves a single cost-effective 3-day pre-differentiation procedure. The pre-differentiated neurons are post-mitotic and can be subplated onto poly-D-lysine/laminin-coated microplates with high consistency. Differentiation of i³Neurons does not require viral infection, puromycin selection, or frequent medium change, thereby reducing the possibility of introducing variability and contamination during HTS. Thus, standardized HTS procedures can be established regardless of location, time, or users, facilitating future use of i³Neurons for ultra-HTS of large libraries of chemical compounds.

Tau-targeted approaches have been proposed as an alternative therapeutic strategy for AD and other tauopathies (Dehdashti et al., 2013; DeVos et al., 2013; Panza et al., 2016). Previous tau-targeted small-molecule strategies—including kinase inhibitors against hyperphosphorylated tau, tau aggregation inhibitors, microtubule stabilizers, and compounds that enhance clearance of tau aggregates—have had limited success (Brunden et al., 2009; Gruninger, 2015; Medda et al., 2016; Min et al., 2015; Panza et al., 2016). Lowering total tau levels may have beneficial effects. For example, the MAPT H1c haplotype increases tau expression and is associated with increased risk of progressive supranuclear palsy, corticobasal degeneration, and AD (Baker et al., 1999; Di Maria et al., 2000; Myers et al., 2005, 2007). Tau lowering in transgenic mouse models rescued functional deficits and tau-mediated neurodegeneration (Andrews-Zwilling et al., 2010; Ittner et al., 2010; Lasagna-Reeves et al., 2016; Min et al., 2015; Roberson et al., 2007, 2011; Vossel et al., 2010) and appears to be safe (Morris et al., 2013). However, discovering compounds that reduce total tau levels has been challenging. Tau is natively unstructured and has proved a difficult target for rational drug design. Several tau-lowering candidates that reduce tau gene transcription were identified by small-scale screening with in-cell western analysis (Dickey et al., 2006). AlphaLISA and homogeneous time-resolved fluorescence assays were used in SH-SY5Y cells to screen for tau-lowering compounds (Dehdashti et al., 2013). How-

ever, these studies were done and validated only in tumor cell lines that lack well-defined axons highly enriched in tau. Previous tau-lowering compounds also caused significant cytotoxicity (Dehdashti et al., 2013). In addition, the regulation of tau homeostasis may differ in rodent and human neurons. Indeed, methylene blue, which reduces the levels of human tau overexpressed in mouse neurons *in vivo* and *in vitro* (Congdon et al., 2012; Hosokawa et al., 2012), had minor effect in human neurons in our assay.

Using i³Neurons in a robust HCS assay, we identified AR agonists as a class of tau-lowering compounds. Both α - and β -AR signaling appeared to regulate tau levels in similar fashion, as AR activation led to tau reduction and AR inhibition led to tau accumulation. Besides moxonidine hydrochloride (α -adrenergic agonist) and metaproterenol hemisulfate (β -adrenergic agonist), three other α - or β -adrenergic agonists (clonidine, dexmedetomidine, and isoproterenol) also reduced tau levels in human neurons. The tau-lowering effects of moxonidine and metaproterenol were abolished by their corresponding antagonists atipomezole and propranolol, which elevated tau levels by themselves. In agreement with our findings, the selective β 2-AR antagonist ICI 118,551 increased tau phosphorylation and accumulation in an AD mouse model (Branca et al., 2014). Paradoxically, genetic suppression of β 2-ARs reduced tau pathology (Wisely et al., 2014). One likely explanation could be that complete removal of β 2 AR prevented harmful effects of dysregulated β 2-ARs, such as binding to A β .

Deficiency in α - or β -AR signaling has been implicated in AD. Levels of high-affinity α 2-ARs are markedly reduced in AD patients (Pascual et al., 1992). Polymorphisms of β -ARs are linked to increased risk of late-onset AD (Yu et al., 2008). The natural ligand of ARs is norepinephrine, whose major source in the CNS is noradrenergic neurons in the locus coeruleus (LC); these neurons project widely to the forebrain, which includes two regions especially affected by tauopathies, the hippocampus and neocortex (Mather and Harley, 2016). Patients with AD have significant degeneration of neurons in the LC and much lower levels of norepinephrine. Interestingly, abnormal tau lesions emerged predominantly in LC regions even in individuals in their 20s, 30s, and 40s (Braak and Del Trecidi, 2015; Braak et al., 2011), suggesting that low levels of norepinephrine in the LC may lead to tau accumulation in young susceptible people and can eventually propagate to other brain regions.

How adrenergic signaling affects tau homeostasis is not known. Interestingly, some AR-modulating compounds may have a beneficial effect that is independent of their AR agonist activities. For example, norepinephrine and isoproterenol, whose chemical backbones contain 1,2-dihydroxybenzene, reduce insoluble tau levels by directly binding cysteine residues in tau to prevent tau oligomerization, independent of their AR agonist activities (Soeda



et al., 2015). Modulating adrenergic signaling could affect other aspects of AD pathology as well. Chronic activation of β -ARs increases A β production (Ni et al., 2006) and protects against the detrimental effects of A β on hippocampal function (Li et al., 2013). AR activation also reduces lipopolysaccharide-induced expression of tumor necrosis factor alpha in microglia (Schlachetzki et al., 2010; Szabo et al., 1997) and interferon- γ -induced expression of class II antigens in astrocytes (Frohman et al., 1988). More studies are needed to dissect the molecular mechanisms underlying the role of adrenergic signaling in AD and to further validate AR agonists as a potential therapeutic strategy for AD and related tauopathies.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

All medium, reagents, and supplements for iPSC culture and differentiation were from Invitrogen unless otherwise specified. Doxycycline, DMSO, cytosine β -D-arabinofuranoside (Ara-C), salicylate, LOPAC library, AR agonists and antagonists, and electrophysiology related chemicals were from Sigma.

Generating and Selecting i^3 N iPSC Clones

Ngn2 transgene was subcloned to a pUCM donor vector containing an AAVS1 homology arm. The Tet-ON 3G-controlled *Ngn2* transgene was integrated to the AAVS1 locus of human iPSC lines through a TALEN nuclease pair. Genomic DNA from puromycin-selected and expanded clones were purified and genotyped by three PCR reactions. We generated i^3 N iPSC lines from two independent wild-type genetic background human iPSC lines: WTC11 (Miyaoka et al., 2014) and F12486.13 (female, white, age at biopsy 48 years, reprogrammed by Sendai virus, generated by Dr. Celeste Karch, Washington University in St. Louis). The iPSC protocol was approved by the Committee on Human Research at the University of California, San Francisco (15-15798). A detailed protocol is described in [Supplemental Experimental Procedures](#).

i^3 Neuron Differentiation

i^3 Neurons were differentiated with a simplified two-step protocol (pre-differentiation and maturation). For pre-differentiation, i^3 N iPSCs were incubated with doxycycline (2 μ g/mL) for 3 days at a density of $2.0\text{--}2.5 \times 10^6$ cells/well in six-well plates coated with Matrigel in knockout Dulbecco's modified Eagle's medium (KO-DMEM)/F12 medium containing N2 supplement, non-essential amino acids (NEAA), mouse laminin (0.2 μ g/mL), brain-derived neurotrophic factor (BDNF, 10 ng/mL), neurotrophin-3 (NT3, 10 ng/mL; Peprotech), and Y-27632. The medium was changed daily, and Y-27632 was removed from day 2. For maturation, pre-differentiated i^3 N precursor cells were dissociated, counted, and subplated at the desired density on plates coated with poly-D-lysine (PDL)/laminin in maturation medium containing 50% DMEM/F12, 50% Neurobasal-A medium, $0.5\times$ B27 supplement, $0.5\times$ N2 supplement, GlutaMax, NEAA, mouse laminin (1 μ g/mL), BDNF (10 ng/mL), and NT3 (10 ng/mL). Half of the

medium was replaced on day 7 and again on day 14, and the medium volume was doubled on day 21. Thereafter, one-third of the medium was replaced weekly until the cells were used. For electrophysiological recording, i^3 N precursor cells were subplated on Matrigel-coated coverslips, and mouse glia were added on day 1 in maturation medium containing 5% heat-inactivated fetal bovine serum and 2 μ M Ara-C.

Immunocytochemistry

i^3 N iPSCs or i^3 Neurons in coverslips were fixed with conditioned medium containing 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated for 1 hr in blocking solution containing PBS, 0.01% Triton X-100, and 5% normal goat serum. The cells were then incubated in blocking solution containing primary antibody overnight at 4°C, followed by incubation with secondary antibody for 1 hr. Images were acquired with an LSM880 confocal system (Zeiss) with Airyscan and a $20\times$ or $63\times$ oil-immersion objective lens. Antibodies used for immunocytochemistry were those against SOX2 (sc-17320S; Santa Cruz Biotechnology), OCT4 (sc-5279; Santa Cruz Biotechnology), TRA-1-81 (sc-21706; Santa Cruz Biotechnology), MAP2 (AB5622 or MAB3418; Millipore), VGlut1 (MAB5502; Millipore), β III tubulin (TUBJ1; Aves Labs), neuronal nuclear antigen (MAB377; Millipore), GABA (A2052; Sigma), HT7 (MN1000; Thermo Fisher), ankyrin G (N106/36; NeuroMa), synapsin-1 (D12G5; Cell Signaling), Olig2 (AB9610; Millipore), GFAP (MAB3402; Millipore), and GluR2/3 (AB1506; Millipore).

Reverse Transcription and Real-Time qPCR

i^3 Neurons were cultured in 96-well PDL plates (655946, Greiner) at a density of 10,000 cells/well for 4 weeks. cDNA from 12 random wells was obtained with Cells-to-CT Kits (Ambion) as recommended by the manufacturer. qPCR reactions were done in duplicate with SYBR Green Real-Time PCR master mixes (Applied Biosystems) and the Applied Biosystems 7900HT fast real-time PCR system. All the primers (Table S1) have been validated with human brain RNA (Zhang et al., 2013). RNAs without reverse transcription were used as negative control, and the dissociation curve from each gene was reviewed to ensure the desired amplification. Expression levels were normalized to *GAPDH*.

HCS Assay to Determine Total Tau Levels

After pre-differentiation, i^3 N precursor cells were placed in 384-well plates at a density of 2,000 cells/well. Fresh maturation medium was added weekly. On day 18, human tau siRNA, known tau-lowering compounds (salicylate, YM-01, and methylene blue) and 1,280 compounds from LOPAC were added and incubated at the desired final concentration for the desired amount of time. Human tau, recognized by HT7 antibody, total neurites, recognized by β III tubulin antibody (TUBJ1; Aves Labs) and nuclei, recognized by Hoechst were detected by a semi-automated immunostaining procedure. A fully automated ArrayScan high-content system (Thermo) was used to acquire images and quantify total tau levels. See [Supplemental Experimental Procedures](#) for detailed methods.

Human Tau and β III Tubulin ELISA

Sensitive human tau and β III tubulin ELISAs were adapted and modified according to previous reports (Barten et al., 2011;



Meredith et al., 2013). Briefly, mouse monoclonal antibody HT7 or rabbit monoclonal β III tubulin antibody (ab68193; Abcam) was used for capture. The respective analytes were detected with alkaline phosphatase-conjugated mouse monoclonal antibodies Tau5 or TUJ1 (806401, 801201; BioLegend). Recombinant full-length human tau (rPeptide) and recombinant β III tubulin (Cytoskeleton) were used to generate standard curves for each assay. The CDP-Star substrate (T2214, Invitrogen) was used as a chemiluminescent alkaline phosphatase substrate. See [Supplemental Experimental Procedures](#) for detailed methods.

Statistics

The sample size for each experiment was determined on the basis of previous experience. Differences between means were assessed by unpaired Student's *t* test (GraphPad Prism, v. 6.0) or multilevel mixed-effects linear regression model (STATA12; StataCorp), as indicated. Values are reported as means \pm SEM. The Shapiro-Wilk test of normality and *F* test to compare variances were applied to datasets when applicable.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2017.08.019>.

AUTHOR CONTRIBUTIONS

L.G., M.E.W., and C.W. conceived the project and designed the experiments. C.W., M.E.W., R.C., K.L., T.E.T., X.C., P.D.S., and C.L. performed the experiments and collected and analyzed the data. M.X., A.M.-F., and D.S. contributed to data analyses and interpretation. C.M.K. generated the original F12486.13 iPSC line. C.W., M.E.W., and L.G. wrote the manuscript.

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